The role of AHR in the regulation of PCSK9 expression and elucidation of its mechanism

Ga Eul Yang

Department of Medical Science

The Graduate School, Yonsei University

The role of AHR in the regulation of PCSK9 expression and elucidation of its mechanism

Directed by Professor Sahng Wook Park

The Master's Thesis

submitted to the Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the

degree of Master of Medical Science

Ga Eul Yang

December 2016

연세대학교
YONSEI UNIVERSITY

This certifies that the Master's Thesis of Ga Eul Yang is approved.

Thesis Supervisor : Sahng Wook Park

Thesis Committee Member#1 : Ho-Geun Yoon

Thesis Committee Member#2 : Sung-Ha Park

The Graduate School
Yonsei University

December 2016

ACKNOWLEDGEMENTS

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마지막으로, 사랑하는 우리 아빠, 엄마, 찬연이. 늘 고맙고 사랑합니다.

양 가 을 올림

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ABSTRACT

The role of AHR in the regulation of PCSK9 expression and elucidation of its mechanism

Ga Eul Yang

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor **Sahng Wook Park**)

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that promotes degradation of the low density lipoprotein (LDL) receptor on the cell surface. Recent studies on PCSK9 inhibition have provided solid evidences on its importance as a target of hypercholesterolemia treatment.

To develop PCSK9 inhibitors as an anti-hypercholesterolemic therapeutic modality, the regulatory mechanism of PCSK9 expression should be elucidated in thorough detail. Although several studies have suggested that transcription factors including sterol regulatory element-binding protein 2 and

hepatocyte nuclear factor 1 modulate the expression of PCSK9 at the transcriptional level, yet little is reported about pathophysiologic conditions that require the control of changes in PCSK9 expression.

In this study, it was shown that aryl hydrocarbon receptor (AHR) is one of transcriptional regulators of PCSK9 expression according to cellular changes in cholesterol metabolism. AHR is a ligand-activated transcription factor that binds to the xenobiotic response element (XRE) in promoters of target genes under cytoprotective and inflammatory conditions. Recently, the role of AHR in adipogenesis and obesity has been reported.

Activation of AHR by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and β-naphthoflavone (BNF), well-established AHR agonists, effectively reduced the expression of PCSK9 mRNA and protein level, resulting in increased the uptake of fluorescence-labeled LDL particles into HepG2 cells. The proximal promoter of *PCSK9* gene harboring XREs was activated accordingly by treatment of TCDD and BNF. In contrast, knockdown of the *AHR* gene by siRNAs increased PCSK9 expression in HepG2 cells, while effects of AHR agonists on PCSK9 expression were impeded by *AHR* knockdown.

These findings suggest that AHR is a transcription factor regulating the expression of PCSK9 under the condition that needs to save the metabolic requirement of *de novo* cholesterol biosynthesis, such as inflammation and

stress conditions. In addition, AHR may be the potential therapeutic target of hypercholesterolemia treatment by way of PCSK9 down-regulation.

Key words : PCSK9, LDLR, AHR, hypercholesterolemia, transcription factor

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I. INTRODUCTION

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is indentified as a member of the proteinase K subfamily of subtilisin serine proteases and regulates cholesterol homeostasis by promoting degradation of the low density lipoprotein (LDL) receptor.[1-3] The hepatic LDL receptor on the cell surface is

a key determinant of the concentration of plasma cholesterol by uptaking LDL-cholesterol from blood into hepatocytes .[4,5] PCSK9 binds to epidermal growth factor-like repeat A (EGF-A) domains of the LDL receptor, and the LDL receptor/PCSK9 complex enters the cell via receptor-mediated endocytosis.[6-8] The internalized complex is then metabolized via lysosomal degradation. That is, Increase in the PCSK9 level leads to increase in the plasma LDL-cholesterol level by reducing the amount of the LDL receptor on hepatocytes and the LDL-cholesterol clearance.[8,9]

Several genetic mutations of PCSK9 in human have been reported in relation with abnormal plasma LDL-cholesterol levels. Gain-of-function mutation of PCSK9 leads to decline in the amount of LDL receptor, resulting in an increase of plasma LDL cholesterol level.[10] Thus, Gain-of-function mutation of PCSK9 is associated with autosomal-dominant familial hypercholesterolemia and cardiovascular disease.[10-12] On the contrary, Loss-of-function mutation of PCSK9 leads to decrease in degradation of LDL receptor and increase in uptake of LDL cholesterol, resulting in elevating LDL-cholesterol clearance.[7,10,11,13]

Statin drugs are the most widely prescribed medicine for treatment of hypercholesterolemia. Statin works as a inhibitor of 3-hydroxy-3-methyl-

glutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of biosynthesis of cholesterol.[14-16] Inhibition of HMA-CoA reductase by statins causes reduction of the intracellular concentration of cholesterol and leads to increase in the uptake of LDL-cholesterol via the increased LDL receptor driven by activation of , sterol-regulatory element binding protein 2 (SREBP-2) .[14,17,18]

Paradoxically, statins increases the expression of PCSK9 expression, a target of SREBP-2.[19] PCSK9 has been reported as a gene that is regulated transcriptionally by SREBP-2 and hepatocyte nuclear factor 1(HNF-1).[19,20] In this study, I have focused on searching another regulatory factors that participates in PCSK9-mediated cellular cholesterol metabolism, particularly, under conditions as of inflammation and oxidative stresses, that requires increased cholesterol supply.

Recently, several studies have reported that Aryl hydrocarbon receptor (AHR), a target of Nuclear factor (erythroid-derived 2)-like 2 (NRF2), is associated with inflammation and cholesterol biosynthesis.[21-24]

AHR, is a ligand-activated transcription factor belonging to basic helix-loop-helix (bHLH)/per-Arnt-Sim (PAS) family, and an important regulator of gene expression related to xenobiotic metabolism.[21,25] It has a high affinity to ligands containing a number of hydrophobic environmental contaminants

which have halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons. The most well-known AHR agonists are 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and β-naphthoflavone (BNF) [26] which activate the pathway involved in xenobiotic metabolism.[21,22,27,28] Under the unstressed condition, AHR resides in the cytoplasm as a inactive form, while activated AHR-ligand complex translocates into the nucleus, binds aryl hydrocarbon nuclear translocator (ARNT), and forms AHR-ARNT heterodimer.[21] AHR-ARNT heterodimer recognizes and binds specifically to the DNA recognition sites named as dioxin response element (DRE) or xenobiotic response element (XRE), of which the consensus sequence are 5'-GCGTG-3' on promoter regions of target genes.[21,22,28] Accordingly, ligand-activated AHR induces the expression of xenobiotic target genes such as cytochrome P450S (CYPs) CYP1A1, CYP1A2 and CYP1B1. These proteins are primarily associated with xenobiotic and drug metabolism.[22,28] AHR-ARNT complex dissociates a after target gene regulation, and AHR translocates back to cytosol from nucleus, then AHR is degraded in proteasome.[29]

Interestingly, when AHR is overexpressed without exposure to a ligand, AHR did not participate in up-regulation, however, it promotes nuclear RelA,

the nuclear factor-κB (NF κB) subunit, and cytosolic interlukin-6 (IL-6) expression in human lung cells.[27]

The purpose of this study is to determine a new mechanism for modulating transcriptional expression of PCSK9 by AHR. Elucidation of a novel regulatory pathway of PCSK9 expression will be an important support for development of a new potential modality to treat hypercholesterolemia.

II. MATERIALS AND METHODS

1. General methods and supplies

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin sulfate were obtained from Invitrogen (Carlsbad, CA, USA). Delipidated serum (DLPS) was prepared from FBS as described by Hannah et al.[19] Protein concentrations were determined using a BCA kit (Pierce, Rockford, IL, USA). Dil-LDL was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). Other reagents not specified were purchased from Sigma-Aldrich (St.Louis, MO, USA)

2. Cell culture

HepG2 (American Type Culture Collection number HB-8065) cells were maintained in medium A (DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) FBS at 37℃ under a humidified atmosphere of 5% CO_2. For treatment of cells with indicated drugs, HepG2 cells were set up on day 0 (1.5×10^5cells/well in 12 well plate) in medium A supplemented with 10% FBS. On day 1, cells were washed twice with phosphate-buffered saline (PBS) and changed to fresh

medium A supplemented with 10% DLPS. On day 2, cells were switched to fresh medium A supplemented with 10% DLPS and treated with indicated concentration of drugs. 16 h after treatment on day 3, cells were washed twice with PBS and harvested for further analyses.

3. Cell fractionation

Cells were washed with PBS twice and harvested by scraping in cold PBS and collected by centrifugation at 1,000 x g for 5 min at 4°C. The collected cells were frozen in liquid nitrogen and suspended in buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose) with protease inhibitors (1 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF, 2 μg/ml Aprotinin), and stood on ice for 15min. Cells were disrupted by passing 30 times through a 22-G needle using a syringe. For separating nuclei, Cell lysate was centrifuged at 1,000 x g for 7 min at 4℃, and the pellet was used as the nuclear extract, while the supernatant was further processed to prepare the cytosolic and membrane extract. To prepare cytosolic and membrane fractions, supernatant was cleared by centrifugation at 55,000 rpm for 30 min at 4℃. After centrifugation, the supernatant were used as the cytosolic fraction, while the pellet was used to prepare the

membrane fraction after resuspension in lysis buffer (10 mM Tris, 100 mM NaCl, 1% SDS, 1 mM EDTA, 1 mM EGTA) with protease inhibitors and shaken by vortexing at room temperature and saved to membrane fraction. Nuclei were resuspended in buffer C (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 2.5% Glycerol) with protease inhibitors (1 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF, 2 μg/ml Aprotinin) and incubated at 4℃ for 1 hr with rotation. Lysate was cleared by centrifugation at 55,000 rpm for 30 min at 4℃ and the supernatant saved as the nuclear fraction.

4. Immunoblot analysis

Cells were washed twice with ice-cold PBS and lysed with 200 μl of NUN buffer containing 0.33 M NaCl, 1.1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.6), and protease inhibitors by adding directly onto the plate. Cell lysates were harvested and further vortexed at room temperature of 5 min for the complete liberation of proteins. Cell lysates were cleared by centrifugation at 13,200 rpm for 10 min at 4℃. The supernatants were collected as the whole cell lysate. After quantitation of protein, aliquots of proteins were subjected to 10% SDS-PAGE and transferred onto

nitrocellulose ECL membranes (GE healthcare, Piscataway, NJ, USA). Immunoblot analyses were performed using the Supersignal West Pico Chemiluminescent Substrate System (Pierce). The following antibodies were used in the current studies: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-AHR from Cell signaling Technology, Inc. (Beverly, MA, USA); anti-CYP1A1 from Santa Cruz Biotechnology (CA, USA). The polyclonal antibodies against human PCSK9 were prepared as previously described by Jeong et al.[19] The polyclonal antibody against human LDLR was obtained in rabbits using synthetic peptide spanning the C-terminus of the bovine LDLR (amino acids 832-841) as described previously.[19] Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce (Waltham, Massachusetts, USA).

5. Quantitative real-time PCR (RT-PCR)

Total RNA was prepared from HepG2 cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instruction. Removal of DNA from RNA was achieved with RNase-free DNase (Qiagen). cDNA was synthesized from 2 μg of DNase-treated RNA using a High capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using the powerSYBR® Green PCR master (Applied Biosystems) and all reactions were analyzed using the StepOneTM Real-time PCR systems (Applied Biosystems). Each sample was analyzed in duplicate, and the relative amounts of all mRNAs were quantified by the comparative cycle-time method.[19] GAPDH, 36B4 and beta-actin mRNA was used as invariant controls. The primers used for RT-PCR are shown in Table 1.

Table1 . Primers used for real-time PCR

Gene symbol	NCBI Accession No.	Sequence (5' to 3')
PCSK9	NM_174936.3	F: GGCAGGTTGGCAGCTGTTT R: CGTGTAGGCCCCGAGTGT
LDLR	NM_000527.4	F: AGGAGACGTGCTTGTCTGTC R: CTGAGCCGTTGTCGCAGT
AHR	NM_001621.4	F: TGACATCAGACTGCTGAAACC R :CCTGAACTTACAAGAAGGAGAA
CYP1A1	NM_000499.4	F: ATCACTGTGTCTAGCTCCTCT R :TCATTAACATCGTCTTGGACCTC
GAPDH	NM_002046.5	F: GCCCCAGCGTCAAAGGT R: GGCATCCTGGGCTACACTGA

PCSK9, proprotein convertase subtilisin/kexin type 9, LDLR, Low density lipoprotein receptor, AHR, aryl hyrocarbon receptor, CYP1A1, cytochrome P450, family 1, member A1, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, F, forward primer, R, reverse primer

6. Fluorescence microscopy and LDL uptake assay

HepG2 cells were set up on day 0 (1.5×10^5 cells/well in a 12 well plate) in medium A supplemented with 10% FBS. On day 1, cells were washed twice with phosphate-buffered saline (PBS) and changed to medium A supplemented with 10% DLPS and treated with BNF. After 16 hr, cells were washed with PBS and treated with Dil-LDL (Biomedical Technologies, Inc., Stoughton, MA) at the 2 μg/ml concentrations for 2 hr. Cells were washed twice with PBS and fluorescence images were gained by using fluorescence microscope (Olympus, Tokyo, Japan) with rhodamine filter. For quantification of LDL uptake, cells were trypsinized to obtain a single-cell suspension. The mean fluorescence intensities of 10,000 cells were analyzed by fluorescence-activated cell sorting on the FACScan (BD Bioscience, San Jose, CA, USA).

7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay was performed using the kit from Cell signaling Technology, Inc. (Beverly, MA, USA). HepG2 cells were set up on day 0 (1.5×10^6 cells/well in 10 cm plate) in medium A supplemented with 10% FBS. On day 1, cells were washed with PBS twice, changed to

medium A supplemented with 10% DLPS and treated with BNF. On day 2, Cells were treated with formaldehyde for cross-linking proteins to DNA. After adding 1 M glycine to a final concentration of 125mM to stop cross-linking, cells were collected by scraping in ice-cold PBS with protease inhibitors. Cross-linked chromatin was digested with Micrococcal Nuclease to produce DNA-protein fragments with a DNA size in 100-500 bps. Antibodies specific to AHR, Histone H3 rabbit monoclonal antibody, and normal rabbit IgG were added directly to the diluted chromatin preparation for the complex co-precipitates and incubated at 4℃ overnight with rotation. Histone H3 rabbit monoclonal antibody and normal rabbit IgG were used as positive control and negative control, respectively. To capture antibodies, Protein G magnetic beads (Cell signaling technology, Inc., Danvers, Massachusetts, USA) were added at chromatin-antibody complex and incubated at 4℃ with rotation for 2 hr. Cross-links were reversed by adding 5M NaCl and Proteinase K, and incubating 65℃, overnight. After incubation, and DNA was purified and used for PCR amplification. Standard PCR were used to analyze specific DNA sequences where antibodies were bound, with primers to amplify the XRE regions on the *PCSK9* promoter. The primers used for analysis of DNA obtained to ChIP assay are shown in Table 2.

Table 2 . Primers used for analysis of DNA obtained to ChIP assay

XRE sites	Sequence (5' to 3')
XRE mut 1	F: CAGTAGGATTGATTCAGAAGTCTC
	R: GTCAGATTACGCGCAGAGGGAAGA
XRE mut 2	F: TCTGACGCTGTTTGGGGAGGGCGA
	R: TCAGACCCTGAACTGAACGGCGGC
XRE mut 3	F: AGCACCCACACCCTAGAAGGTTTC
	R: TGCAGGAGCTGAAGTTCAGGAGCA

XRE, xenobiotic response element, F, forward, R, reverse

8. Construction of mutant *PCSK9* promoter-reporter plasmids, transient transfection and the luciferase reporter assay

The pGL3-*PCSK9* promoter-reporter construct (D3) was prepared as previously described by Jeong et al.[19] Site-directed mutageneses of xenobiotic response elements (XREs) on the *PCSK9* promoter was performed using *pfu* polymerase (Agilent technologies, Palo Alto, CA, USA) to create mutant variants of the XREs mutant-*PCSK9* promoter-luciferase reporter construct. The primers used for site-directed mutagenesis are shown in Table 3.[22]

Table 3 . Primers used for Site-directed mutagenesis [22]

XRE sites	Sequence (5' to 3')
XRE mut 1	F: GGAGTCTGGCATCCCTATCAGGGTGAGAGGCGG
	R: CCGCCTCTCACCCTGATAGGGATGCCAGACTCC
XRE mut 2	F: ATGGGGCTCTGGTGGATAGATCTGCGCGCCCCA
	R: TGGGGCGCGCAGATCTATCCGCCAGAGCCCCAT
XRE mut 3	F: TCCGCGCGCCCCTTCTATCGCCCTGCTCCTGAA
	R: TTCAGGAGCAGGGCGATAGAAGGGGCGCGCGGA

XRE, xenobiotic response element, the underlines denote the locations where mutant sequences are introduced.

The integrity of construct sequences was confirmed by DNA sequencing. For luciferase reporter assays, HepG2 cells were transiently contransfected in suspension state with the *PCSK9* promoter-reporter construct and pTK-renilla control vector (Promega, Madison, WI, USA) in Opti-MEM (Invitrogen) using Lipofectamine 2000^{TM} (Invitrogen) according to the manufacturer's instruction. Briefly, HepG2 cells were transfected with plasmids on day 0. On day 1, cells were washed twice with PBS and changed to medium A supplemented with 10% DLPS in the presence or absence of the BNF. On day 2, cells were washed twice with PBS, harvested and cell lysates were prepared with passive lysis buffer (Promega). Luciferase activity was analyzed by the Dual-luciferase reporter assay system (Promega) according to the manufacturer's instruction. The firefly luciferase activity was normalized to the renilla luciferase activity and the amounts of protein in the lysate. Assay was done in duplicate and repeated at least three times in independent experiments.

9. Animal experiment

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at Yonsei University Health System. 10- to 12-wk old male C57BL/6J mice purchased from Japan SLC, Ins. (Shizuoka Prefecture, Hamamatsu, Japan) were maintained on 12-h dark/12-h light cycles and fed a standard chow diet (LabDiet, St. Louis, MO, USA) *ad libitum* and had free access to tap water. Mice were injected intraperitoneally at 40 mg/kg/day with the solutions of BNF in DMSO/corn oil [1:7 (v/v)] at 4 mg/ml,[28] for 3 consecutive days at start of the dark cycle. All mice were sacrificed at the end of the dark cycle. After euthanasia, blood was collected in the presence of 2 mM EDTA and aprotinin from inferior vena cava for plasma preparation, and livers were stored at -80℃ until later use. Determination of the concentration of total cholesterol and triacylglycerol, and fast performance liquid chromatography (FPLC) of lipoproteins in plasma were carried out as described previously.[30]

III. RESULTS

1. AHR agonists reduce the expression of PCSK9 protein and mRNA

To determine whether AHR is involved in regulation of the expression of PCSK9, HepG2 cells were treated with AHR agonists, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and β-naphthoflavone (BNF). The changes in the amount of PCSK9 were determined by immunoblot using the PCSK9 antibody. In all researches, the medium was switched from DMEM supplemented with 10% FBS to DMEM supplemented with 10% DLPS (De-lipidated serum) to up-regulate the expression of PCSK9. TCDD (Fig. 1A) and BNF (Fig. 1B) dramatically reduced the expression of PCSK9 compared to a vehicle in HepG2 cells.BNF and TCDD raised expression of AHR target genes such as cytochrome P450S (CYPs) CYP1A1 at indicated concentration. Total RNA was prepared from BNF-treated HepG2 cells for 18 hr and was analyzed with quantitative real-time PCR (Figure 1.C). The amount of mRNAs for *PCSK9* was decreased by BNF, while those for *LDLR* and *AHR* remained unchanged. There was a slight increase in LDLR mRNA level and dramatic increase in *CYP1A1* mRNA level by BNF.

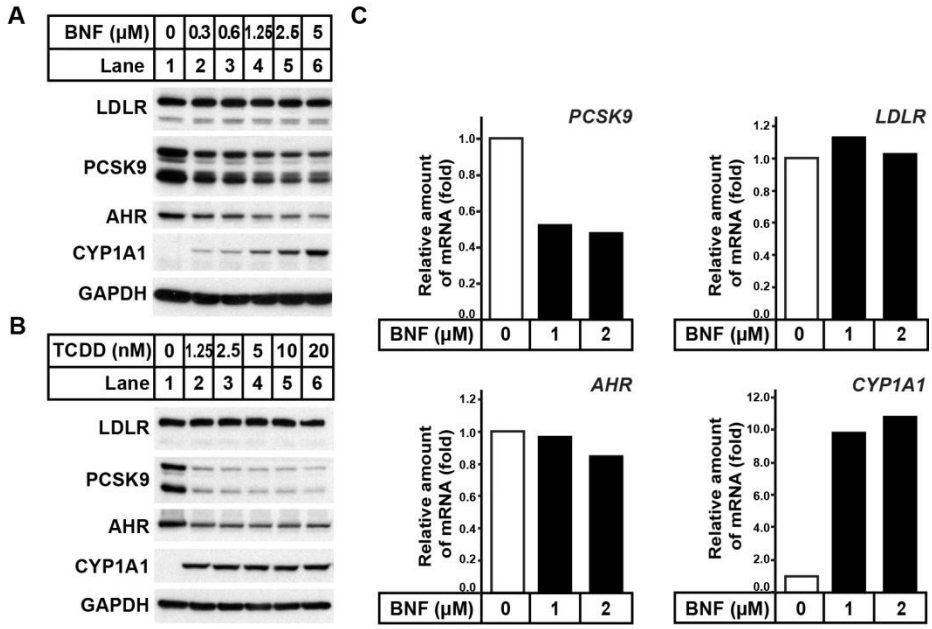

Figure 1. Effects of AHR agonists on PCSK9 and LDLR expression in HepG2 cells. (A and B) HepG2 cells were set up at $1.5x10^5$ cells per well in 12-well plate with DMEM supplemented with 10% FBS on day 0. On day 1, cells were washed twice with PBS, and then pre-incubated with fresh DMEM supplemented with 10% de-lipidated serum (DLPS). On day 2, cells were treated with AHR agonists, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and β-naphthoflavone (BNF) at indicated concentration. After 18 hr incubation, cells were harvested, and whole cell lysates were performed to immunoblot analyses with antibodies against PCSK9, the LDL receptor, AHR, CYP1A1,

GAPDH. GAPDH was used as an invariant control. (C) The effect of BNF on *PCSK9* and *LDLR* mRNA level was analyzed with quantitative real-time PCR. HepG2 cells were treated with BNF and incubated at indicated concentration for 18 hr. Total RNAs were isolated from HepG2 cells and cDNA was synthesized by reverse transcription, and implemented by quantitative real-time PCR. Each value represents the amount of mRNA relative to that in the cells grown with vehicle (DMSO), which is arbitrarily defined as 1. The values represent means from duplicate reactions. β-actin was used as an invariant control.

2. AHR Knockdown increases the expression of PCSK9 in HepG2 cells

To elucidate the regulation of AHR on PCSK9 expression, AHR was knockdown by transient transfection with small interfering RNAs (siRNAs). The siRNA targeting AHR successfully reduced the amount of AHR in HepG2 cells (Figure. 2). AHR knockdown efficiently increased the amount of PCSK9 (Figure. 2A, lane 4) when compared to that with a scrambled control siRNA. (Figure. 2A, lane 1). The effect of BNF (1.0 μM) and TCDD (10 nM) to decrease the amount of PCSK9 (Figure. 2A, lane 2 and 3) were abolished when AHR were knockdown (Figure. 2A, lane 5 and 6). The band intensities of PCSK9 (Figure. 2B) were quantified using Image J software.

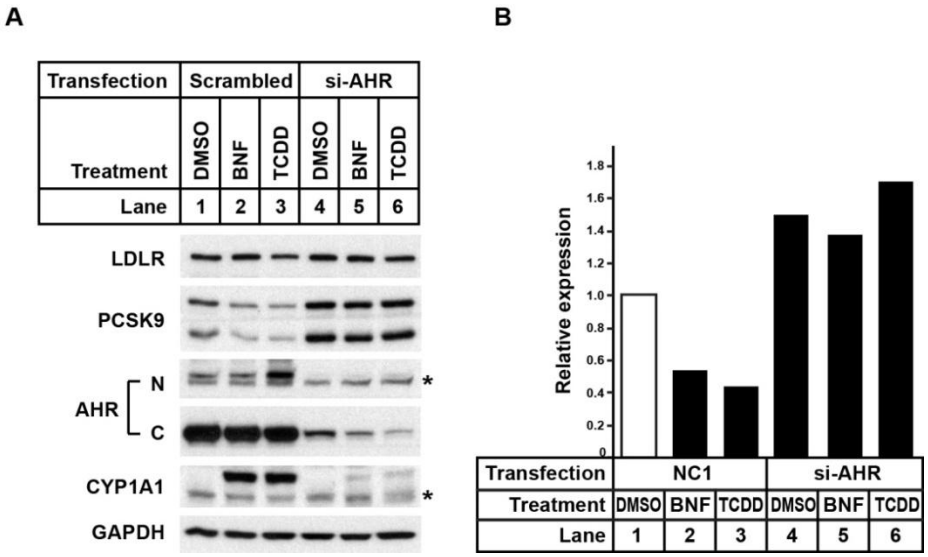

Figure 2. Effects of AHR knockdown on the expression of PCSK9 expression in HepG2 cells. (A) On day 0, HepG2 cells were transiently transfected with negative control (NC1) and si-AHR. On day 1, cells were switched to DMEM with 10% DLPS and treated with BNF (1.0 μM) and TCDD (10 nM). On day2, cells were fractionated as described in materials and methods. N and C for AHR represent the nuclear and cytosolic fraction, respectively. The asterisk indicates irrelevant cross-reacting bands. (B) Analysis of PCSK9 expression was digitized using Image J software. The similar results were obtained in three independent experiments.

3. BNF, a AHR agonist, induces the uptake of LDL-cholesterol

To determine whether the regulation of PCSK9 expression by BNF affects cholesterol homeostasis via the LDL receptor, LDL-cholesterol uptake was assessed. After incubation with DMSO and BNF for 18 hr, Fluorescence-labeled LDL (Dil-LDL) was applied to HepG2 cells and fluorescence microscopy images were obtained. As the expression of the LDL receptor was elevated, cellular LDL-cholesterol uptake and fluorescence intensity were increased (Figure. 3A). Treatment of BNF in HepG2 cells increased the uptake of fluorescence-labeled LDL in a dose-dependent manner (Figure. 3A). Fluorescence in HepG2 cells was quantitated by flow cytometry analyses of cellular mean fluorescence intensity. The data showed as relative fluorescence intensities compared to that of vehicle-treated cells (Figure. 3B). These results suggest that AHR agonist, BNF, down-regulated the expression of PCSK9 and up-regulated the expression of the LDL receptor, resulting in increased uptake of LDL into the cell.

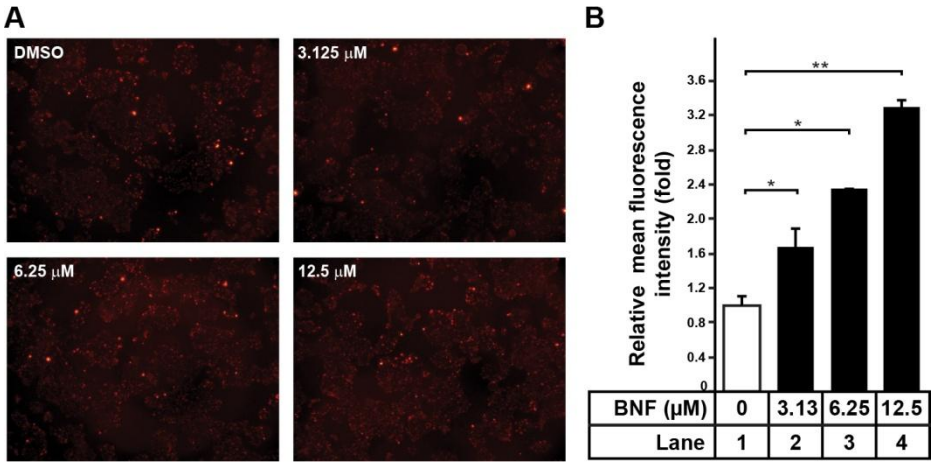

Figure 3. Enhanced uptake of LDL by BNF in HepG2 cells. (A) Fluorescence microscopic images of Dil-LDL uptake in HepG2 cells. HepG2 cells were treated with indicated concentration of BNF for 18 hr, then incubated with 2 μg/ml of Dil-LDL for 3 hr. Fluorescence microscopic images were taken after washing twice with PBS. (B) The relative uptake of fluorescence using FACS analysis. After acquiring microscopic image, cells were harvested by trypsinization, and the mean fluorescence intensity (MFI) of 10,000 cells was measured using the flow cytometry. Each value represents the ratio of the MFI in triplicate reactions relative to that in vehicle-treated cells, which is arbitrarily defined as 1. Error bars represent the SD of triplicate reactions. $*p < 0.05$, $**p < 0.01$ Student's t-test when compared with the values in vehicle-treated cells.

4. BNF Reduces the *PCKS9* Promoter Activity

To evaluate whether the change in the expression of PCSK9 by BNF is regulated at the transcriptional level, the pGL3-PCSK9 promoter-reporter construct was transfected in HepG2 cells transiently, and the luciferase activity was measured. Treatment of BNF in HepG2 cells decreased the *PCSK9* promoter activity in a dose-dependent manner (Figure. 4). These data suggest that AHR after ligand binding activates the transcriptional activity of the *PCSK9* promoter.

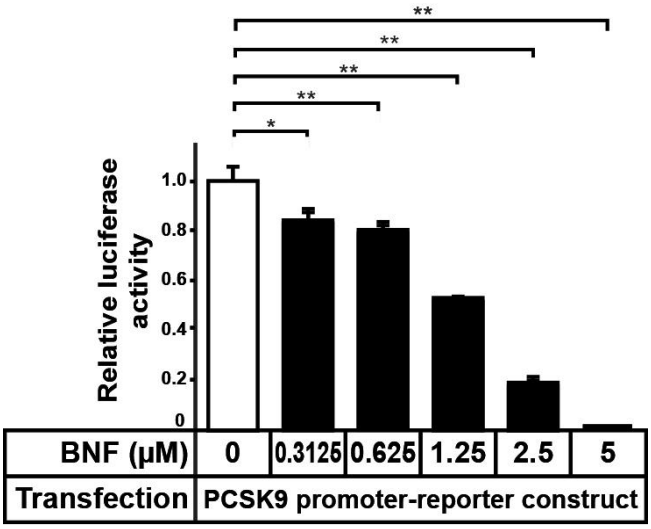

Figure 4. Effects of BNF on the transcriptional activity of the human *PCSK9* promoter in HepG2 cells. After transient transfection of HepG2 cells with the pGL3-*PCSK9* promoter-reporter construct and the pTK-renilla vector as a transfection efficiency control, cells were treated with BNF at indicated concentrations for 18 hr. The luciferase activities were evaluated using the dual-luciferase assay system (Promega). Each value represents the ratio of luciferase activity relative to that in vehicle-treated cells, which is arbitrarily defined as 1. The luciferase activity was normalized with the amounts of protein and transfection efficiency. Error bars represent the SD of triplicate reactions. *$p < 0.05$, **$p < 0.01$ Student's t-test when compared with the values in vehicle-treated cells.

5. AHR binds to xenobiotic response elements (XREs) on the *PCSK9* promoter

To study transcriptional regulation of *PCSK9* by AHR, sequence analysis of the human *PCSK9* promoter spanning from -709 to -94 was carried out. [19] The human *PCSK9* promoter region contains three xenobiotic response elements (XREs) at -542 to -538 (XRE1), -343 to -339 (XRE 2), and -130 to -126 (XRE 3) (Figure. 5A).

To evaluate the function of XREs that mediate the transcriptional regulation of *PCSK9* by AHR, luciferase activity assay was carried out using the pGL3-PCSK9 promoter construct, D3. (Figure. 5B). The luciferase assay driven by the PCSK9 promoter showed that BNF reduced the transcriptional activity of the *PCSK9* promoter significantly. However, the mutation introduced by site-directed mutagenesis at XRE-2 made the reporter construct irresponsive to BNF treatment, while mutations at XRE 1 and XRE 3 site showed responsiveness to BNF the same to that of the wild-type promoter construct. (Figure. 5C). To determine whether AHR binds to XRE sequences in the *PCSK9* promoter, chromatin immunoprecipitation (ChIP) assay using an antibody specific to AHR was carried out. The presence of the XRE sequences in the immunoprecipitated chromatin was examined by PCR using three pairs of primers to target the XREs, XRE 1(-642/-438) ,

XRE 2(-444/-240) and XRE 3 (-318/-98) on the *PCKS9* promoter. ChIP assay showed that only XRE 2 sequences on the *PCSK9* promoter binds to AHR (Figure. 5D). These results suggest that AHR binds to XRE 2 on the *PCSK9* promoter and mediate the transcriptional regulation of *PCSK9* by BNF.

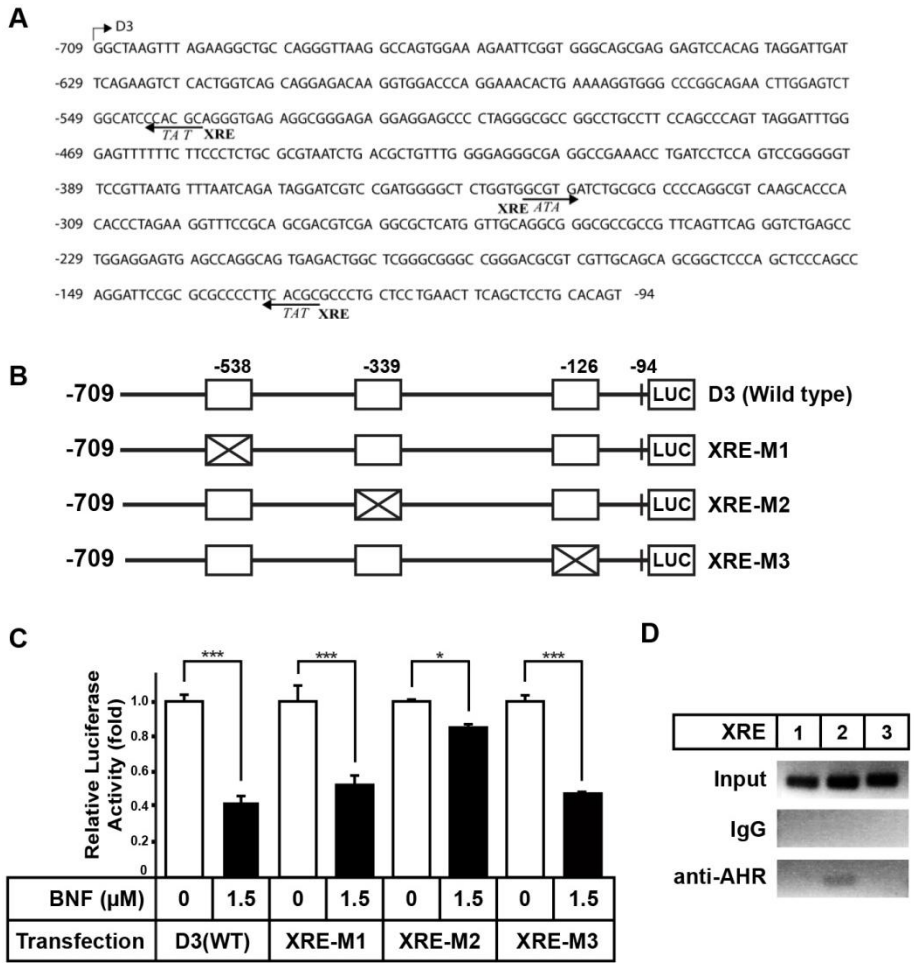

Figure 5. Effects of mutations at XRE1, XRE2, and XRE3 on the role of AHR for the *PCSK9* promoter in HepG2 cells. (A) Nucleotides sequences from-709 to -94 on the *PCSK9* promoter. (B) Mutations were introduced by site-directed mutagenesis of XREs on the PCSK9 promoter region as described under 'Materials and Methods'. (C) HepG2 cells were transiently transfected with the wild-type and mutant PCSK9 promoter-reporter

constructs, and luciferase assay was carried out 18 hr after BNF treatment. Each value represents the ratio of luciferase activity in cells treated with BNF compared to that in cells treated with vehicle. The relative luciferase activity of cells treated with vehicle was arbitrarily defined as 1, regardless of which reporter-construct was transfected into cells. Error bars represent the SD of triplicate reactions. *$p < 0.05$, **$p < 0.01$ Student's t-test when compared with the values in vehicle-treated cells. The similar results were obtained in two independent experiments. (D) Chromosomal DNA from HepG2 cells were extracted and immunoprecipitated with an antibody against AHR. After incubation of the antibody against AHR, the immunoprecipitated DNA were analyzed by PCR using the specific primers to XREs.

6. BNF reduced the concentration of plasma total cholesterol and LDL-cholesterol in mice

To evaluate the *in vivo* effect of BNF in mice, 10 - 12 wk-old male mice were injected with BNF or vehicle (n=5 per group). Mice were injected intraperitoneally daily at 40 mg/kg/day of BNF suspended in DMSO/corn oil [1:7 (v/v)] for 3 consecutive days. Table 4 lists parameters of mice injected with BNF. The plasma concentration of total cholesterol in mice injected with BNF decreased significantly by 27% compared to that in control mice injected with DMSO. To determine the changes in lipoprotein fractions by BNF, lipoprotein profile of the pooled plasma way analyzed by using FPLC. The amount of cholesterol in LDL fractions in mice injected with BNF decreased significantly compared to that in mice injected with vehicle (Figure 6, fraction numbers 13-19). These results suggest that BNF reduced the increased uptake of LDL into the cell, resulting in reducing plasma concentration of cholesterol in mice. In addition, BNF reduced the amount of cholesterol in fractions containing high-density lipoprotein (Figure. 6, fraction numbers 20-28).

Table 4. The Effect of BNF in Wild-Type Mice

Parameter	Vehicle	BNF
No. & Sex	5 males	5 males
Body weight (g)	24.9 ± 0.3	24.4 ± 0.2
Liver weight (g)	1.31 ± 0.02	1.39 ± 0.03
Liver wt. / body wt. (%)	5.28 ± 0.05	5.71 ± 0.15
Plasma cholesterol (mg/dL)	108 ± 5.9	79 ± 5.0*
Plasma TG (mg/dL)	37 ± 2.26	29 ± 5.87*

Male mice, 10-12 wk of age, were fed a chow diet. Mice were injected at 40 mg/kg/day with BNF or vehicle (DMSO), for 3 consecutive days. Each value represents the mean ± S.E. of the indicated number of mice. Asterisks denote the level of statistical significance (Student's *t* test). *, $P < 0.05$. The similar results were observed in three independent experiments.

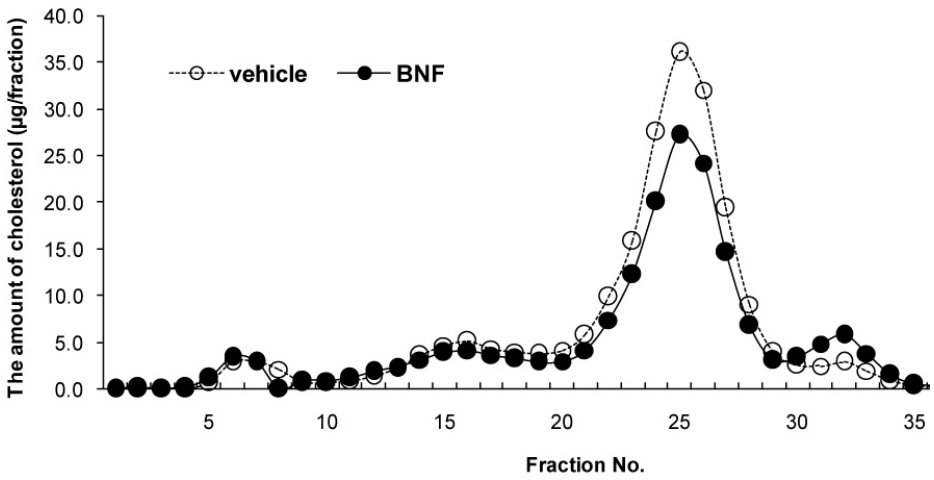

Figure 6. Lipoprotein profile of the pooled plasma of wild-type mice treated with BNF. The pooled plasma from mice described in Table 4 was analysed by using FPLC to determine the changes in lipoprotein fractions in mice injected with BNF or vehicle. The amount of cholesterol in each fraction was determined as described under 'Materials and Methods'. The similar results were observed in 3 independent experiments.

IV. DISCUSSION

Although cholesterol is a fundamental component of cells to maintain biological life, [31,32] the increased level of the plasma LDL cholesterol, which is primarily modulated by the LDL receptor on the cell surface, is well-reported to cause cardiovascular diseases and atherosclerosis.[33]

The expression of the LDL receptor is transcriptionally increased by SREBP-2, a key transcription factor that regulates overall genes participating *de novo* cholesterol biosynthesis in cells. Paradoxically, the expression of *PCSK9*, which functions to counteract the role of the LDL receptor, increases simultaneously by SREBP-2.[34] Thus, inhibition of PCSK9 expression is an important target to reduce the plasma concentration of cholesterol by increasing the uptake of LDL cholesterol mediated by up-regulation of the amount of LDL receptor. Up to date, PCSK9 expression has been reported to be modulated by transcription factors such as SREBP-2 and HNF-1.[19,20] The purpose of this study was to elucidate a novel mechanism of transcriptional regulation of PCSK9 to provide the basic concept for development of PCSK9 inhibitors. Based on several previous studies in my laboratory, AHR was proposed as a putative transcription factor which is involved in the regulation of PCSK expression. AHR has been reported to be associated with obesity and lipid metabolism.[21,22] AHR is an cytosolic transcriptional factor that binds to

ligands such as BNF and TCDD, well-known environmental contaminants. Ligand-activated AHR translocated into nuclei from cytosol and binds to aryl hydrocarbon nuclear translocator (ARNT) and forms AHR-ARNT complex. AHR-ARNT complex binds specific DNA recognition site known as xenobiotic response element (XRE), and induces xenobiotic metabolism. BNF and TCDD induce detoxification enzymes as CYPs.

In this study, the amount of PCSK9 protein and mRNA were decreased in a dose-dependent manner by BNF and TCDD, resulting in increased uptake of LDL-cholesterol in HepG2 cells. In addition, BNF reduced transcriptional activity of the PCSK9 promoter in HepG2 cells. To elucidate whether this regulation is achieved at transcriptional level of the *PCSK9* promoter by AHR, the PCSK9 promoter region was analyzed for prediction of transcription factor binding sites. It revealed that the human PCSK9 promoter region spanning from -709 to -94 contains three xenobiotic response elements (XREs).[22] Three XREs were located at XRE 1 (-542 to -538) , XRE 2 (-343 to -339) and XRE 3 (-130 to -126) on PCKS9 promoter-reporter construct (D3). Mutational assay and ChIP assay showed that the luciferase assay indicated that luciferase activity driven by XRE 1 element-mutant and XRE 3 element-mutant elements have no effect on BNF compared to that in that by XRE 2 element-mutant which remained slight changed by BNF. The interaction of AHR to the

XRE 2 was affirmed by Chromatin immunoprecipitation assay .These results suggest that AHR is an important modulator as the transcriptional expression of PCSK9 by binding XRE element 2 on *PCSK9* promoter.

In addition, mice were injected with BNF to evaluate AHR regulate uptake of LDL cholesterol by suppression of PCSK9 expression *in vivo*. BNF activates AHR and significantly lowers the plasma concentration of cholesterol in mice injected BNF compared to mice injected with DMSO. As a result, AHR regulates the expression of PCSK9, resulting in increase the amount of the LDL receptor and uptake of LDL-cholesterol *in vivo* and *in vitro*.

In summary, this study suggests that AHR is a transcription factor modulating the expression of PCSK9 in cholesterol metabolism and AHR may be the potential therapeutic target of cardiovascular disease and atherosclerosis.

V. CONCLUSION

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that promotes the degradation of the LDL receptor on the cell surface. Inhibition of PCSK9 has been reported to be a potent target of hypercholesterolemia treatment. In this study, it was supposed that AHR is one of the regulators of PCSK9 expression in relation with changes in cholesterol metabolism. Activation of AHR by TCDD and BNF, well-known AHR agonists, reduced the expression of PCSK9 protein and mRNA levels, resulting in increased uptake of LDL particles into the HepG2 cells. The proximal promoter of PCSK9 harboring XREs was activated by BNF accordingly. In contrast, knockdown of the AHR gene by siRNA increased the expression of PCSK9 in HepG2 cells, while effects of AHR agonists were impeded by AHR knockdown. *In vivo* administration of BNF in wild-type mice significantly reduced the level of plasma total cholesterol, and slightly LDL fractions. These findings strongly suggest that AHR is a transcription factor regulating the expression of PCSK9 under the condition that requires saving the metabolic requirement of *de novo* cholesterol biosynthesis. In addition, AHR may be a potential therapeutic target of hypercholesterolemia treatment.

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ABSTRACT (IN KOREA)

PCSK9 발현 조절인자로서 **AHR**의 역할 및 작용 기전 규명

<지도교수 **박 상 욱**>

연세대학교 대학원 의과학과

양 가 을

Proprotein convertase subtilison kexin 9 (PCSK9)는 세포막에 존재하는 Low density lipoprotein (LDL) receptor와 결합하여 이를 세포 내로 유입시킨 후 분해를 촉진시키는 단백질이다. PCSK9의 발현 증가로 인한 LDL receptor의 발현 감소는 혈중 LDL-콜레스테의 농도 증가를 초래하게 되고, 이에 따른 동맥경화 등 심혈관계 질환의 발생이 증가한다. 따라서 PCSK9는 발현 혹은 활성을 억제함으로써 혈중 콜레스테롤의 농도 감소를 유도 할 수 있고 심혈관계 질환의 발생을 줄일 수 있는 고톨레스테롤혈증 치료제 개발의 중요한 표적 단백질이다.

PCSK9 억제제의 개발이 이루어지기 위해서는 PCSK9의 발현 조절이 어떠한 기전을 통하여 이루어지는 지 밝히는 것이 매우 중요한 요인이다. 현재까지 SREBP2와 HNF-1등의 전사인자가 PCSK9의 발현을 조절하는 주요 인자로 보고되었으나, 아직까지 체내 콜레스테롤의 항상성 유지와 관련된 구체적인 조절 기전은 밝혀져 있지 않다. 본 연구에서는 최근 지질대사 조절 인자로서 그 연관성이 보고된 aryl hydrocarbon receptor (AHR)가 PCSK9의 발현을 조절 할 수 있는 중요한 전사인자일 것으로 추론하고 그 작용 기전을 밝히고자 하였다. AHR는 환경오염물질에 노출되었을 주어졌을 때 세포의 생존과 연관된 유전자의 발현을 조절하는 전사인자로 알려져 있다. 세포가 환경물질에 되었을 때, Aryl hydrocarbon receptor(AHR)은 Aryl hydrocarbon nuclear translocator(ARNT)와 결합하여 전사인자로써 표적유전자 promoter 부위의 xenotbiotic response element(XRE)에 결합한다.

si-RNA를 이용하여 AHR을 저 발현 시켰을 때 PCSK9의 발현이 증가되었다. 세포에 AHR을 활성화 시킬 수 있는 2,3,7,8 - tetrachlorodibenzo-*p*-dioxin (TCDD) and β-naphthoflavone (BNF)을 처리했을 때 PCSK9의 단백질 및 mRNA의 발현이 감소됨을 확인하였고, AHR의 활성이 PCSK9 프로모터의 전사 활성을 감소함을 밝혔다. AHR에 의한 PCSK9 프로모터의 전사 억제기전을 밝히고자 AHR의 결합

하는 XRE 부위의 돌연변이 유도 실험을 진행하였고, AHR이 특이적으로 PCSK9 프로모터의 2번째 XRE 부위에 결합함을 확인하였다. 또한, BNF를 세포에 처리했을 때 세포 내로 LDL 콜레스테롤 유입이 증가하는 것을 확인하였으며, 동물에 BNF를 투여했을 때에도 혈중 콜레스테롤의 감소를 확인하였다.

따라서, 이러한 결과를 통해 AHR에 의한 PCSK9의 발현 조절 기전을 밝히고, AHR의 활성화 물질을 이용하여 PCSK9 발현 억제를 확인하여 향후 PCSK9 억제제 개발 가능성을 제시하였다.

핵심되는말 : 고콜레스테롤혈증, PCSK9, LDLR, AHR